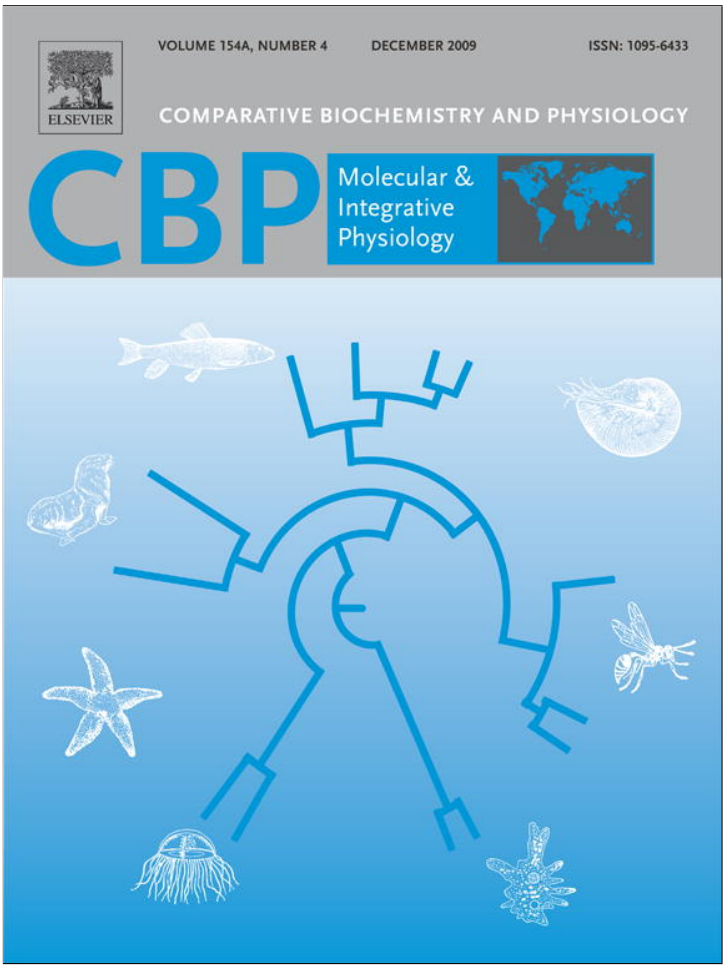

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Calcium regulation in wild populations of a freshwater cartilaginous fish, the lake sturgeon *Acipenser fulvescens*

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ABSTRACT

Lake sturgeon, *Acipenser fulvescens*, are one of a few species of cartilaginous fishes that complete their life cycle entirely in freshwater. Sturgeons maintain very low concentrations of circulating calcium (Ca^{2+}) compared with other vertebrates, and therefore, face unique challenges in regard to Ca^{2+} regulation, which are likely to be magnified during vitellogenic stages of the reproductive cycle. In the present study, Ca^{2+} concentrations and associated hormones of female and male lake sturgeon were examined in two wild populations, and were related to reproductive stage. In both populations, free, bound and total Ca^{2+} were low, peaking in mid-late vitellogenic females. Internal Ca^{2+} and phosphate (PO_4^{3-}) concentrations were inversely related to environmental concentrations, suggesting that these ions are preferentially retained and that mechanisms for mobilization are up-regulated under diminished environmental concentrations. Plasma 17β-estradiol, 11-ketotestosterone and testosterone, peaked in mid-late vitellogenic females, while the androgens peaked in spawning males. Urine Ca^{2+} was more tightly regulated than other divalent ions and decreased in spawning fish. Therefore, the increases in free plasma Ca^{2+}, the very low circulating concentrations of free and total Ca^{2+}, and the increase in PO_4^{3-} and bound Ca^{2+} in low Ca^{2+} environments indicate unique adaptations to Ca^{2+} regulation in the lake sturgeon.

1. Introduction

The importance of regulating plasma calcium (Ca^{2+}) concentration within tight limits is well recognized in all vertebrates. Free or ionic Ca^{2+} has direct effects on numerous physiological functions with maladaptive changes incurring from immediate and long-term disruptions of homeostasis (Bushinsky and Monk, 1998). Hypocalcemia can result in increased neuromuscular excitability, leading to tetany (Pang et al., 1971; Pang, 1973; Bushinsky and Monk, 1998), whereas hypercalcemia may depress neuromuscular excitability and lead to muscle weakness (Diercks et al., 2004). Long-term disruptions of Ca^{2+} homeostasis in vertebrates with a calcified skeleton can compromise bone integrity due to the reduction in available stores of Ca^{2+} (Nordin and Morris, 1989).

In aquatic environments, fishes take Ca^{2+} up through their gills and across intestinal epithelia with the kidneys playing a role in the overall regulation of the plasma concentration of the ion. Bony fishes have an added advantage over those fishes with a cartilaginous skeleton, in that bony fish have an internal Ca^{2+} store in their skeleton and scales. Indeed, it has been proposed that this internal store was one mechanism that allowed bony fish to radiate from Ca^{2+} rich (≥10 mM) seawater environments to low Ca^{2+} (≤0.1 mM) and ion poor freshwater (FW) environments. By comparison, there are few examples of cartilaginous fishes occupying FW environments. The potamotrygonid rays exclusive to the Amazon basin are one example and the chondrosteans, such as sturgeons and paddlefish which are found in the Northern hemisphere, are another example. Research on FW Ca^{2+} regulation has been investigated to a small degree in elasmobranchs (Smith and Smith, 1931; Urist, 1962; Thorson, 1967; Thorson et al., 1967; Piermarini and Evans, 1998), but is very limited in primitive fishes such as chondrosteans (Fuentes et al., 2007). Although most sturgeons occupy marine habitats at some point in their life cycle, several species, including the lake sturgeon, *Acipenser fulvescens*, found within the Great Lakes and Hudson Bay drainage basins in North America, occupy FW throughout their life cycle. Interestingly, plasma Ca^{2+} concentration in sturgeons has been reported as one of the lowest in the vertebrate phyla (Urist and Van de Putte, 1967; Urist et al., 1972). These reportedly low levels in plasma Ca^{2+} concentration in combination with the lack of a bony

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skeleton and an entirely FW existence suggest that sturgeons are operating close to a lower physiological threshold, and may be susceptible to changes in environmental Ca^{2+}.

In all vertebrates, and particularly females, the demands for Ca^{2+} are exacerbated during the reproductive cycle. In fishes, vitellogenin (VTG), a yolk-precursor protein, is synthesized in the liver following stimulation by 17β-estradiol (E_2). VTG, a lipoglycophosphoprotein, requires Ca^{2+} in its production, and because of its polar phosphate (PO_4^{3-}) groups, binds large amounts of Ca^{2+} and other divalent cations such as magnesium (Mg^{2+}) in the plasma (Mommsen and Walsh, 1988). After transit through the systemic vasculature, the VTG molecule is sequestered into developing oocytes, effectively removing Ca^{2+} from maternal stores. These high demands for Ca^{2+} must be met by external uptake or internal mobilization, or a combination thereof. External uptake occurs primarily through the gills in FW fishes, although the gut may also play a role (Flik et al., 1995). Internal uptake is primarily from the scales in teleost fish with acellular bone, however, teleosts with cellular bone are known to utilize skeletal structures as an additional calcium store (Yamada et al., 2002).

Interestingly, female lake sturgeon have a protracted reproductive cycle, and maintain an iteroparous, high-reproductive-output life history strategy that results in a considerable input of Ca^{2+} into developing oocytes. Furthermore, in terms of available Ca^{2+} stores, lake sturgeon are known to actually have regressive or smaller ganoid scales or scutes with age (Findeis, 1997; Peterson et al., 2007). Thus, the available mineral pool of Ca^{2+} would seem to diminish at the very time when it would be required for reproductive development. How a cartilaginous fish is able to handle this loss of Ca^{2+} and how it regulates Ca^{2+} during the reproductive cycle are not well understood.

Therefore, in the present study, Ca^{2+} and associated ion concentrations in the plasma and urine were examined during the reproductive cycle of the lake sturgeon. Plasma E_2, 11-ketotestosterone (11-KT), and testosterone (T) were related to reproductive stage and Ca^{2+} regulation. Calcified structures such as the ganoid scales and fin rays were examined to determine their role as potential internal Ca^{2+} stores. We hypothesized that circulating levels of Ca^{2+} would be closely linked to changes in sex steroid levels and therefore reproductive state in adult lake sturgeon. Further, we have tested the impact of different environments on this relationship by examining two distinct populations of lake sturgeon.

2. Materials and methods

2.1. Field sampling

Wild fish were captured in 2007 and 2008 from the Winnipeg River near Pinawa, Manitoba, Canada between Slave Falls and Seven Sisters hydropower dams, and from the Lake Winnebago system (Lake Winnebago and Lake Poygon) near Oshkosh, Wisconsin, USA. Non-lethal capture of fish in the Winnipeg River was conducted using gill nets ranging in size from 203 to 305 mm, during the ice-free period from May to October. In Wisconsin, fish were sampled in February as a part of the winter recreational spear-fishery. Samples were obtained from these fish as they were brought to registration stations associated with the fishery.

In the Winnipeg River, fish were quickly removed from the gill net in the morning (overnight set), and placed into a large ($2 \times 1 \times 1.2$ m) live-well supplied by a bilge-pump with flow-through river water. Fish were anesthetized in a separate container using clove oil (2 mL clove oil: 15 mL 70% ethanol: 40 L of river water; Peake 1998) until gill opercular movements dramatically slowed and a hand inserted into the mouth failed to stimulate clamping of the palatal complex. Fish were quickly placed in a supine position in a nylon-mesh (1.5-m cradle section) fish stretcher, and gills were irrigated with river water from a submersible pump connected to a vinyl tube inserted into the mouth. A blood sample was quickly collected from the caudal vasculature immediately posterior to the anal fin via a 20-gauge hypodermic needle and 3 mL heparinized vacutainer. A urine sample was collected using a 3 cc syringe connected to an approximately 20 cm section of polyethylene cannula tubing (1 mm ID) which was inserted through the urogenital opening into the urinary papillae. Blood samples were immediately measured for hematocrit by centrifuging at 12,600 ×*g* for 3 min. Both blood and urine samples were then centrifuged at 5000 ×*g* for 3 min, and the plasma or supernatant were removed, placed in separate vials and frozen in liquid nitrogen on the boat. These samples were then stored at −80 °C until further analyses. While blood and urine were being sampled, a 3–5 cm incision was made via a scalpel 1–1.5 cm to the side of the ventral mid-line, about two-thirds of the distance from the pectoral fins to the anus. Using Allis forceps and a biopsy instrument, a sample of gonad tissue was taken. Gonad tissue was then immediately placed in a 1:50 *w/v* 10% neutrally phosphate buffered methanol-free-formalin fixative. The opening was then sealed with 4–5 sutures, the fish was weighed and measured for fork and total length, and tagged with a spaghetti-style tag at the base of the dorsal fin. The fish was then allowed to recover from the procedure on board the boat prior to release at the site of capture.

In the Lake Winnebago system, fish were sampled when brought by recreational spear-fishers to state fishery checkpoints on or immediately adjacent to the lakes. Fish that arrived at the weigh stations frozen (air temperatures ranged from 5 to −20 °C) were not sampled. Blood was collected through the caudal vasculature or via the conus arteriosus using an 18 gauge needle and a heparinized 3 mL syringe or vacutainer. Blood and gonadal biopsy samples were processed as described above. Urine could not be collected due to freezing of the urogenital opening, and freezing within the cannula tubing. The first two lateral scutes on the right side of the fish and one of the leading pectoral fin rays were also removed for Ca^{2+}-content analysis.

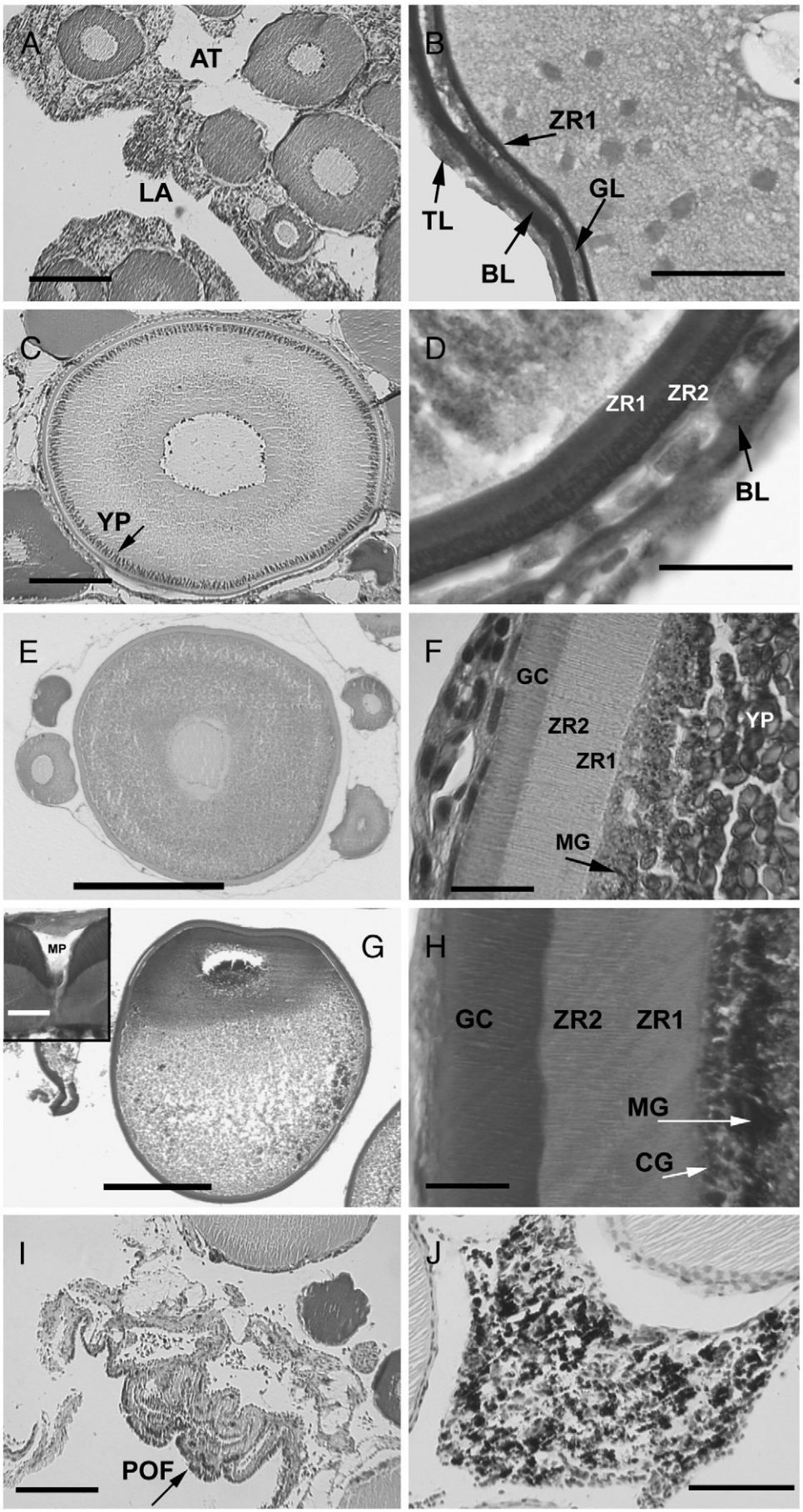
2.2. Water chemistry

Data accessed from Environment Canada (B. Chu, Regina, Saskatchewan, personal correspondence) and the U.S. Geological Service (USGS, 2009) were used to compare water chemistry (Ca^{2+}, PO_4^{3-}, pH) between the two sampling locations. For the Winnipeg River, data from three different river locations nearby the sampling site were used: 1966–1974 data for Slave Falls, 1972–2006 data for Pointe du Bois, and 1960–1974 data for Pine Falls. For the Lake Winnebago system, data from 1988–2003 for two locations upriver from Lake Winnebago (Fox River at Berlin, Wolf River at New London), and two locations in or immediately downriver of Lake Winnebago (Fox River at Appleton and Lake Winnebago outlet at Neenah-Menasha) were used.

2.3. Blood plasma and urine ion analyses

Plasma osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Inc., Logan, Utah, USA). Plasma sodium (Na^+), potassium (K^+), Ca^{2+}, Mg^{2+}, fluoride (F^-), chloride (Cl^-), bromide (Br^-), sulfate (SO_4^{2-}) and PO_4^{3-} were measured by ion-exchange chromatography (Metrohm-Peak, Herisau, Switzerland). The cation eluent was 4 mM tartaric acid and 0.75 mM dipicolinic acid, and the anion eluent was 3.6 mM Na_2CO_3 with CO_2 suppression

Fig. 1. Female lake sturgeon reproductive stages. Pre-vitellogenic (A, B), early vitellogenic (C, D), mid-late vitellogenic (E, F), fully mature/spawning (G, H), and post-spawn (I, J). Abbreviations: AT = adipose tissue, BL = basal lamina, CG = cortical granules, GC = gelatinous coat, GL = granulosa layer, LA = lamellae, MG = melanin granules, MP = micropyle, POF = post-ovulatory follicle, TL = thecal layer, YP = yolk platelets, ZR = zona radiata. Bar = 10 μm (G inset), 20 um (B, D, F, H), 100 μm (J), 200 μm (A, C, I), 1000 μm (E, G).



by 100 mM H_2SO_4 followed by CO_2 free air. Total Ca^{2+} was measured by atomic absorption spectrometry (AA240FS, Varian Inc., Palo Alto, CA, USA) using 0.1% HNO_3 as a diluent, and was independently verified using an inductively coupled plasma optical emission spectrometer (725-ES, Varian Inc.) using 2% HNO_3 as a diluent. Bound calcium was calculated as total minus free Ca^{2+}. All assays were run in duplicate or triplicate.

2.4. Blood plasma sex steroid analyses

The plasma sex steroids E_2, T and 11-KT were measured by radioimmunoassay as described by Webb et al. (2002). Each sample was run in duplicate, and the lower limit of detection for each assay was 1.25 ng/mL. The average recovery efficiencies were 87% for T, 87% for 11-KT, and 81% for E_2. The intra and interassay coefficients of variation for all assays were less than 5% and 10%, respectively. Steroid levels (determined by radioimmunoassay) were validated by verifying that serial dilutions were parallel to standard curves. Values below the limit of detectability were considered to be zero for statistical analyses.

2.5. Scute and fin ray Ca^{2+} content

Scutes and fin rays were stored at −20 °C prior to analyses. Samples were thawed, boiled in water for 1–2 min, adhering tissue was removed, and then dried at 60 °C for 2 days. Samples were weighed (nearest 0.0001 g), and then digested in 10 volumes (*w*/*v*) of 2 N HNO_3 at 60 °C for 5 days in sealed 50-mL polyethylene vials. Samples were vortexed daily to increase the rate of digestion. Ca^{2+} content, calculated from the digests with the assumption of a volume (mL) to mass (g) ratio of approximately one, was determined using atomic absorption spectrometry, as described above. All samples were run in triplicate.

2.6. Histological methods

Gonad samples that were previously fixed were dehydrated in an ethanol gradient, treated with a clearing agent, infiltrated and embedded in paraffin, sectioned at 6 μm, floated on a heated water bath and mounted to albumin-coated glass slides. Glass slides were dried on a slide warmer overnight (40 °C), paraffin was removed with a clearing agent, tissue was rehydrated in an ethanol gradient and then stained with either hematoxylin and eosin or Alcian blue-periodic acid Schiff (PAS) counterstained with hematoxylin. The PAS stain differentiates glycoprotein structures (egg chorion and yolk platelets). Cover slips were adhered to the stained sections with permount mounting media (Fisher Scientific, Ottawa, Ontario, Canada).

2.7. Stages of gonadal development

Stages of gonadal development were modified from Bruch et al. (2001) and based on histological assessment similar to that for Atlantic sturgeon, *A. oxyrinchus* (Van Eenennaam and Doroshov, 1998).

2.7.1. Females

Pre-vitellogenic (Fig. 1A, B). Small, translucent to white oocytes; the first zona radiata may be present, but there are no yolk platelets in the basophilic cytoplasm.

Early vitellogenic (Fig. 1C, D). In the largest oocytes, yolk platelets are present in the cytoplasm: the 2nd layer of the chorion may be present.

Mid-late vitellogenic (Fig. 1E, F). The 3rd layer of the chorion is present, and pigmentation varies in intensity. The yolk may be polarized, with small platelets in the animal hemisphere and large platelets at the vegetal hemisphere.

Spawning/mature oocytes (Fig. 1G, H). The nucleus has migrated into the egg cortex near the animal pole and numerous cortical granules can be seen in a layer near the animal pole.

Post-spawn (Fig. 1I, J). This stage follows spawning and is characterized by post-ovulatory follicles, atretic bodies and pre-vitellogenic oocytes.

2.7.2. Males

Immature (Fig. 2A, B). The testicular tissue contains mitotically dividing gonial cells enclosed in cysts.

Maturing (Fig. 2C, D). Many of the testicular cysts have initiated meiosis, and contain primary and secondary spermatocytes, spermatids and spermatozoa.

Spawning (Fig. 2E, F). The testicular cysts and ducts contain differentiated spermatozoa.

2.7.2.1. Statistical analysis. Data was $\log_{10}$ transformed when necessary to meet parametric assumptions of normality and homogeneity of variance. One-way analysis of variance (ANOVA) was run for each parameter (ions, sex steroids, etc.) with reproductive state, analyzing sexes and populations separately. Tukey's multiple comparison tests were used to compare means following a significant ANOVA result. Student's *t*-tests were run when there were only two reproductive groups, such as for Wisconsin males and for pooled scute samples. When data could not be transformed to meet ANOVA assumptions, non-parametric Kruskal–Wallis tests followed by Dunn's multiple comparison tests were used. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Reproductive stages

In the Winnipeg River, there were a total of 7 to 16 females from five different reproductive stages, and 8 to 20 males from three reproductive stages sampled (Table 1). In the Lake Winnebago system, a total of 5 to 27 females from five reproductive stages and 24 to 25 males from two reproductive stages were sampled. Because fish were sampled at one time of the year in the Lake Winnebago system, only two stages of males were encountered. There were no differences in length between the female reproductive stages or male reproductive stages, respectively, from the Winnipeg River. In the Lake Winnebago system, lengths of pre-vitellogenic females and immature males were shorter than more developed stages. Fish in the Winnipeg River tended to be smaller at each reproductive stage than those from the Lake Winnebago system.

3.2. Water chemistry

Water in the Winnipeg River had lower Ca^{2+} and PO_4^{3-} concentrations and pH than water from the Lake Winnebago system (Table 2). Ca^{2+} was approximately 2.5 times lower and PO_4^{3-} was approximately 4 times lower in the Winnipeg River, whereas, pH was relatively high in the Lake Winnebago system at 8.33.

3.3. Blood plasma Ca^{2+}, PO_4^{3-} and associated ion concentrations

Ionic or free plasma Ca^{2+} increased over two-fold between pre-vitellogenic and mid-late vitellogenic females from the Winnipeg River (Fig. 3A). In contrast, plasma free Ca^{2+} concentrations were only elevated a small amount in fish that were vitellogenic in the Lake Winnebago system, and the different stages of vitellogenesis could not be distinguished by plasma Ca^{2+} concentration (Fig. 3B). Bound Ca^{2+} displayed a very similar pattern to free Ca^{2+} for both populations

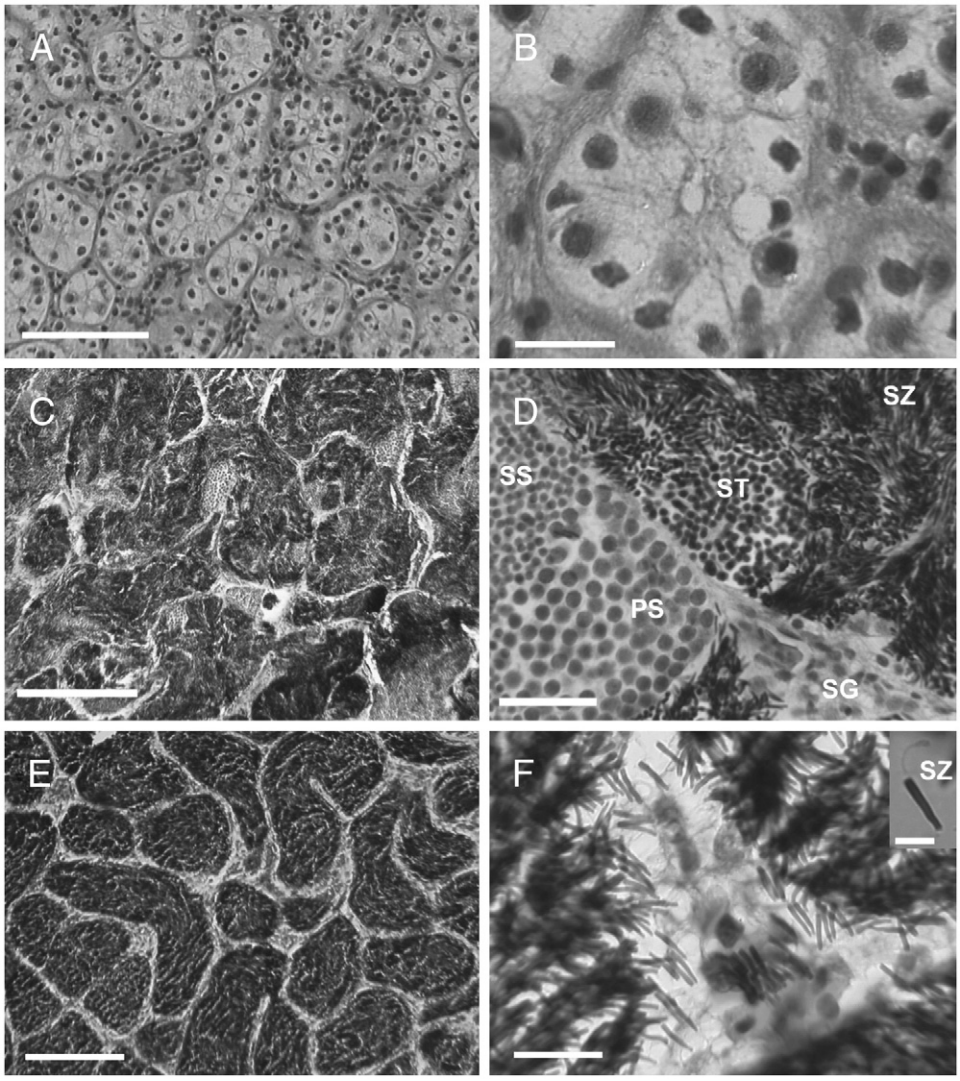


Fig. 2. Male lake sturgeon reproductive stages. Immature (A, B), maturing (C, D), and spawning (E, F). Abbreviations: PS = primary spermatocyte, SG = spermatogonia, SS = secondary spermatocyte, ST = spermatid, SZ = spermatozoa. Bar = 5 μm (F inset), 20 um (B, F), 25 μm (D), 100 um (A), 200 μm (C, E).

Table 1
Reproductive stages and mean lengths (±S.E.) of lake sturgeon captured in the Winnipeg River, MB, Canada, and the Lake Winnebago system in WI, USA.

Location	Sex	R. stage	*n*	FL (cm)	TL (cm)
MB	F	PV	8	120.7 ± 3.7	130.5 ± 4.3
MB	F	EV	7	125.5 ± 5.6	137.1 ± 6.7
MB	F	M-LV	13	126.5 ± 4.0	138.2 ± 4.5
MB	F	SP	16	130.4 ± 3.5	141.6 ± 4.0
MB	F	PS	7	126.3 ± 9.5	137.3 ± 9.6
MB	M	IM	10	106.0 ± 3.0	114.9 ± 3.2
MB	M	MA	8	112.3 ± 3.5	121.3 ± 3.1
MB	M	SP	20	106.8 ± 2.7	116.9 ± 2.7
WI	F	PV	27	125.9 ± 3.7^{a}	137.0 ± 3.8^{a}
WI	F	EV	14	148.6 ± 1.7^{b}	160.5 ± 1.8^{b}
WI	F	M-LV	5	159.9 ± 4.0^{b}	172.2 ± 4.1^{b}
WI	F	SP	11	162.7 ± 3.7^{b}	175.1 ± 3.8^{b}
WI	F	PS	10	148.0 ± 3.1^{b}	159.9 ± 3.3^{b}
WI	M	IM	25	117.4 ± 3.1^{A}	128.2 ± 3.2^{A}
WI	M	SP	24	139.1 ± 2.9^{B}	150.6 ± 3.0^{B}

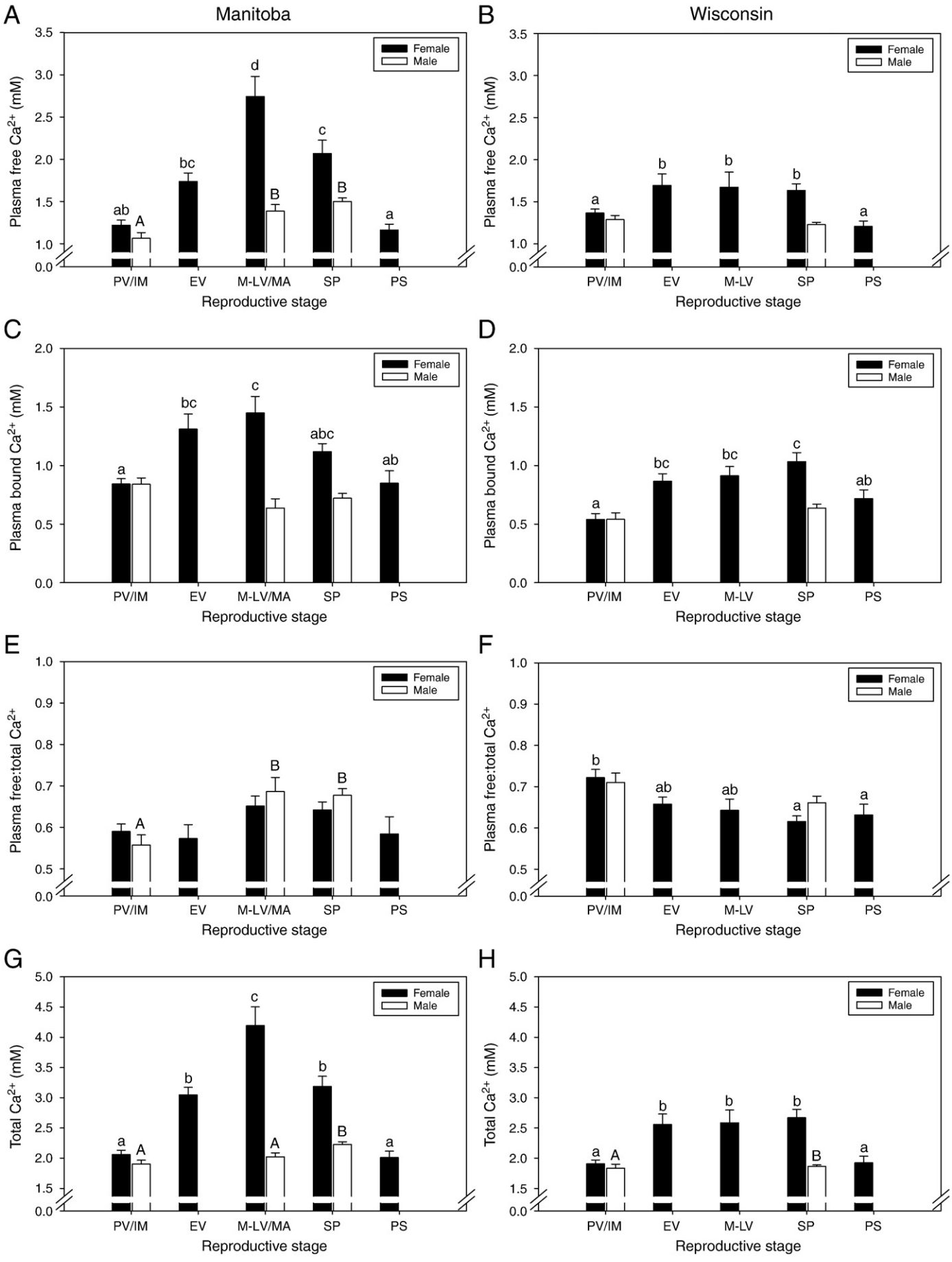
MB = Manitoba, WI = Wisconsin, R = reproductive, FL = fork length, TL = total length, F = female, M = male, PV = pre-vitellogenic, EV = early vitellogenic, M-LV = mid-late vitellogenic, SP = spawning, PS = post-spawn, IM = immature, MA = maturing. Different superscript lowercase or uppercase letters indicate significant ($P < 0.05$) differences between female or male reproductive groups, respectively. One-way ANOVA, Tukey's multiple comparison tests, or if ANOVA assumptions were could not be met by transformation of data: non-parametric Kruskall–Wallis with Dunn's multiple comparison tests.

(Fig. 3C, D). The ratio of free:total Ca^{2+} was regulated at a level that was not different between female reproductive groups in the Winnipeg River fish, although males had higher ratios at later reproductive developmental stages (Fig. 3E). In contrast, fish from the Lake Winnebago system had higher levels of free:total Ca^{2+} at early reproductive stages than at later reproductive stages for both females and males (Fig. 3F). Total Ca^{2+}, combining both free and bound Ca^{2+}, clearly differentiated female reproductive stages in the Winnipeg River fish (Fig. 3G). Male fish had similar concentrations to pre-vitellogenic and post-spawn females, although spawning fish had slightly higher concentrations than other male reproductive groups. In the Lake Winnebago system, plasma total Ca^{2+} was elevated in vitellogenic fish and remained high in spawning females (Fig. 3H), but was still much lower than mid-late vitellogenic fish from the Winnipeg

Table 2
Water chemistry in the Winnipeg River, Manitoba, Canada and the Lake Winnebago system in Wisconsin, USA.

Drainage	Ca^{2+} (mM)	PO_4^{3-} (nM)	pH
Winnipeg River, MB	0.35 ± 0.01	137 ± 43	7.47 ± 0.08
Lake Winnebago system, WI	0.87 ± 0.04	572 ± 168	8.33 ± 0.35

Data compiled from Environment Canada and the U.S. Geological Survey (USGS 2009). Values are means ± S.E.



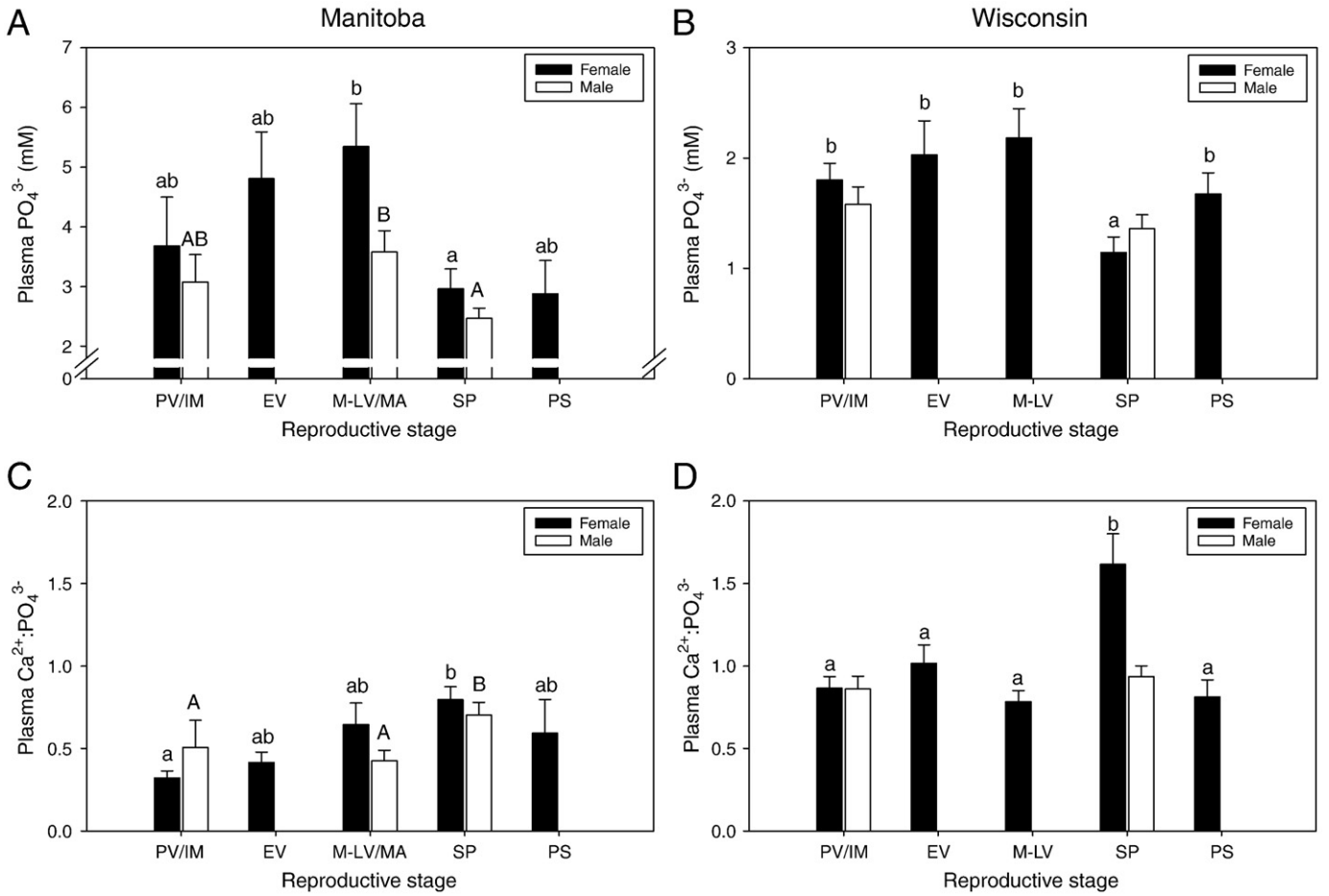


Fig. 4. Mean (+S.E.) plasma PO_4^{3-} (A, B) and Ca^{2+}: PO_4^{3-} (C, D) in lake sturgeon captured in the Winnipeg River, Manitoba, Canada and Lake Winnebago system, Wisconsin, USA. Different superscript lowercase or uppercase letters indicate significant differences between female (black bars) or male (white bars) reproductive groups, respectively. One-way ANOVA, Tukey's multiple comparison tests, except for Manitoba males: non-parametric Kruskall–Wallis with Dunn's multiple comparison tests, $P<0.05$, $n=5$–27. Note the *y*-axis values change from A to B.

River. Interestingly, plasma total Ca^{2+} in fully mature/spawning males was slightly elevated above mid-spermatogenic (maturing) males, similar to the Winnipeg River.

Plasma PO_4^{3-} showed similar trends for both the Winnipeg River and Lake Winnebago system fish, with concentrations increasing with reproductive developmental stage until spawning and post-spawning stages, where the lowest concentrations were found (Fig. 4A, B). However, the concentrations of plasma PO_4^{3-} were much higher in the Winnipeg River fish than in the Lake Winnebago system fish. The ratio of plasma Ca^{2+}:PO_4^{3-} which is generally closely correlated in vertebrates, showed very different patterns between the two populations (Fig. 4C, D). In the Winnipeg River fish, plasma PO_4^{3-} was present at greater concentrations than Ca^{2+}, whereas in the Lake Winnebago system fish, Ca^{2+} and PO_4^{3-} were generally at very similar concentrations. In females from both populations, and males from the Winnipeg River, the ratio of plasma Ca^{2+}:PO_4^{3-} peaked at spawning, due to decreased PO_4^{3-} concentrations.

For other ions, plasma F^- concentrations were greater in mid-late vitellogenic female fish compared to non-vitellogenic fish (pre-vitellogenic and post-spawn) from the Winnipeg River although fish from the Lake Winnebago system did not show this pattern (Table 3). Plasma Cl^-, SO_4^{2-} and K^+ did not change with reproductive stage in either population. Plasma Br^- decreased in spawning fish in the Winnipeg River, but did not change in the Lake Winnebago system. Plasma Na^+ did not change with reproductive stage in the Winnipeg River, but was greater in early vitellogenic and spawning fish than pre-vitellogenic fish in the Lake Winnebago system. Plasma Mg^{2+} was greater in spawning fish than pre-vitellogenic fish in the Winnipeg River, but not different between reproductive stages in the Lake Winnebago system. Plasma osmolality was greater in mid-late vitellogenic fish than spawning fish in the Winnipeg River although it was not different at any reproductive stage in Wisconsin. Blood hematocrit did not change with reproductive stage in either population, but was greater at every reproductive stage in the Winnipeg River fish.

In males, plasma F^- was greater in maturing fish than in immature or spawning fish in the Winnipeg River, but was not different between reproductive stages in the Lake Winnebago system (Table 4). Male plasma Cl^-, SO_4^{2-}, K^+ and osmolality were not different between reproductive stages in either population. Plasma Br^- decreased in spawning fish in the Winnipeg River but did not change in Wisconsin. Plasma Na^+ was greater in maturing males than in immature males in the Winnipeg River and greater in spawning than immature males in the Lake Winnebago system. Plasma Mg^{2+} increased in spawning fish

Fig. 3. Mean (+S.E.) plasma free (A, B), bound (C, D), free:bound (E, F), and total Ca^{2+} (G, H) in lake sturgeon captured in the Winnipeg River, Manitoba, Canada (A, C, E, G) and Lake Winnebago system, Wisconsin, USA (B, D, F, H). Different lowercase or uppercase letters indicate significant differences between female (black bars) or male (white bars) reproductive groups, respectively (One-way ANOVA, $P<0.05$, Tukey's multiple comparison tests, $n=5$–27).

Table 3
Mean (±S.E.) plasma (P) and urine (U) ion concentrations (mM), osmolality (mmol/kg) and hematocrit (%) in wild female lake sturgeon captured in the Winnipeg River, Manitoba, Canada and Lake Winnebago system, Wisconsin, USA.

Ion	Location	Type	PV	EV	M-LV	SP	PS
F^-	WI	P	0.05 ± 0.03	0.39 ± 0.17	0.54 ± 0.02	0.17 ± 0.06	0.58 ± 0.28
	MB	P	1.36 ± 0.89^{a}	9.67 ± 5.13^{ab}	12.32 ± 4.42^{b}	2.32 ± 0.37^{ab}	0.67 ± 0.55^{a}
	MB	U	6.85 ± 1.11^{ab}	11.53 ± 3.20^{b}	9.45 ± 2.57^{b}	2.81 ± 0.50^{a}	6.44 ± 3.43^{ab}
Cl^-	WI	P	122.7 ± 1.5	127.6 ± 0.8	124.7 ± 1.4	124.6 ± 2.6	128.2 ± 1.5
	MB	P	120.1 ± 3.1	128.6 ± 10.4	131.8 ± 5.2	118.3 ± 2.4	118.1 ± 8.3
	MB	U	3.8 ± 0.8	8.7 ± 2.7	5.9 ± 1.3	9.9 ± 2.7	9.2 ± 3.3
Br^-	WI	P	0.024 ± 0.004	0.024 ± 0.005	0.011 ± 0.002	0.022 ± 0.003	0.019 ± 0.002
	MB	P	0.097 ± 0.007^{b}	0.124 ± 0.012^{b}	0.130 ± 0.011^{b}	0.074 ± 0.003^{a}	0.089 ± 0.010^{ab}
	MB	U	0.002 ± 0.001^{a}	0.010 ± 0.004^{ab}	0.009 ± 0.005^{ab}	0.019 ± 0.004^{b}	0.010 ± 0.004^{ab}
SO_4^{2-}	WI	P	1.87 ± 0.12	2.36 ± 0.18	2.27 ± 0.26	2.45 ± 0.22	2.27 ± 0.24
	MB	P	2.16 ± 0.12	1.96 ± 0.11	2.40 ± 0.16	1.94 ± 0.09	1.86 ± 0.29
	MB	U	2.59 ± 0.51	3.94 ± 0.61	4.61 ± 0.93	2.29 ± 0.14	2.70 ± 0.76
Na^+	WI	P	137.3 ± 1.4^{a}	144.9 ± 1.8^{b}	141.6 ± 3.3^{ab}	148.8 ± 2.5^{b}	144.9 ± 2.8^{ab}
	MB	P	136.5 ± 3.7	138.6 ± 2.7	140.4 ± 3.5	136.1 ± 1.9	135.5 ± 4.3
	MB	U	20.0 ± 2.4	25.1 ± 2.6	20.8 ± 2.5	19.0 ± 2.7	25.6 ± 6.6
K^+	WI	P	2.45 ± 0.19	2.91 ± 0.16	2.40 ± 0.08	2.22 ± 0.14	2.90 ± 0.21
	MB	P	1.93 ± 0.18	2.25 ± 0.17	2.32 ± 0.18	1.95 ± 0.11	1.94 ± 0.25
	MB	U	2.79 ± 0.45	4.68 ± 0.74	4.33 ± 0.59	3.29 ± 0.34	3.77 ± 0.61
Mg^{2+}	WI	P	0.98 ± 0.03	1.10 ± 0.04	0.97 ± 0.03	1.01 ± 0.07	1.04 ± 0.06
	MB	P	0.58 ± 0.05^{a}	0.81 ± 0.03^{ab}	1.02 ± 0.07^{bc}	1.07 ± 0.04^{c}	0.77 ± 0.06^{ab}
	MB	U	1.48 ± 0.28^{ab}	2.27 ± 0.61^{b}	2.14 ± 0.52^{b}	0.86 ± 0.05^{a}	1.65 ± 0.37^{ab}
Osmolality	WI	P	286.6 ± 2.4	288.2 ± 2.0	291.0 ± 3.2	289.5 ± 3.4	283.9 ± 2.1
	MB	P	283.5 ± 5.3^{ab}	287.7 ± 3.5^{ab}	291.8 ± 3.1^{b}	279.3 ± 2.5^{a}	278.7 ± 2.5^{ab}
	MB	U	56.6 ± 6.1	79.9 ± 12.1	72.5 ± 10.9	58.6 ± 5.1	64.3 ± 15.4
Hct %	WI	B	19.8 ± 1.3	17.3 ± 1.9	19.6 ± 1.2	17.5 ± 3.1	14.8 ± 2.0
	MB	B	31.9 ± 2.1	33.4 ± 1.4	32.7 ± 1.4	33.5 ± 1.1	30.5 ± 3.1

PV = pre-vitellogenic, EV = early vitellogenic, M-LV = mid-late vitellogenic, SP = spawning, PS = post-spawn, MB = Manitoba, WI = Wisconsin, B = whole blood. Different superscript lowercase letters indicate significant differences between reproductive groups. One-way ANOVA, Tukey's multiple comparison tests, or if ANOVA assumptions were could not be met by transformation of data: non-parametric Kruskall–Wallis with Dunn's multiple comparison tests, $P<0.05$, $n=5$–27.

in the Winnipeg River, similar to females, but decreased in spawning fish in the Lake Winnebago system. Plasma osmolality did not change in either population, but blood hematocrit was greater in spawning fish than immature fish in the Winnipeg River, and greater at all reproductive stages compared to the Lake Winnebago system.

Table 4
Mean (±S.E.) plasma (P) and urine (U) ion concentrations (mM), osmolality (mmol/kg) and hematocrit (%) in wild male lake sturgeon captured in the Winnipeg River, Manitoba, Canada and Lake Winnebago system, Wisconsin, USA.

Ion	Location	Type	IM	MA	SP
F	WI	P	0.22 ± 0.12		0.26 ± 0.10
	MB	P	5.11 ± 2.27^{A}	7.69 ± 1.16^{B}	2.65 ± 0.52^{A}
	MB	U	8.13 ± 3.08	8.21 ± 1.38	
Cl^-	WI	P	123.6 ± 0.9		124.0 ± 1.3
	MB	P	126.2 ± 5.3	123.0 ± 5.4	115.7 ± 2.1
	MB	U	10.7 ± 5.4	6.7 ± 1.7	
Br^-	WI	P	0.019 ± 0.002		0.030 ± 0.004
	MB	P	0.105 ± 0.009^{B}	0.125 ± 0.005^{B}	0.082 ± 0.004^{A}
	MB	U	0.006 ± 0.004	0.006 ± 0.002	
SO_4^{2-}	WI	P	1.92 ± 0.16		2.21 ± 0.17
	MB	P	1.77 ± 0.17	2.11 ± 0.12	1.87 ± 0.14
	MB	U	3.39 ± 0.82	4.05 ± 0.31	
Na^+	WI	P	138.3 ± 1.46^{A}		145.3 ± 1.35^{B}
	MB	P	132.6 ± 2.0^{A}	143.1 ± 4.2^{B}	134.4 ± 1.8^{AB}
	MB	U	23.1 ± 5.2	23.8 ± 2.9	
K^+	WI	P	2.69 ± 0.19		2.33 ± 0.16
	MB	P	1.70 ± 0.13	1.96 ± 0.14	1.94 ± 0.11
	MB	U	3.41 ± 0.58^{A}	6.30 ± 0.50^{B}	
Mg^{2+}	WI	P	0.99 ± 0.03^{A}		0.83 ± 0.04^{B}
	MB	P	0.68 ± 0.06^{A}	0.76 ± 0.06^{A}	1.02 ± 0.03^{B}
	MB	U	1.54 ± 0.38	1.64 ± 0.12	
Osmolality	WI	P	282.4 ± 2.2		288.0 ± 2.1
	MB	P	278.7 ± 5.1	287.8 ± 3.8	281.0 ± 2.5
	MB	U	64.7 ± 5.4	73.4 ± 6.1	
Hct %	WI	B	18.9 ± 1.3		23.3 ± 2.3
	MB	B	30.9 ± 1.4^{A}	36.1 ± 1.3^{AB}	36.7 ± 1.1^{B}

PS = post-spawn, IM = immature, MA = maturing, SP = spawning, MB = Manitoba, WI = Wisconsin, B = whole blood. Different uppercase letters indicate significant differences between reproductive groups. One-way ANOVA, Tukey's multiple comparison tests, or if ANOVA assumptions were could not be met by transformation of data: non-parametric Kruskall–Wallis with Dunn's multiple comparison tests, $P<0.05$, $n=8$–25.

3.4. Urine Ca^{2+}, PO_4^{3-} and associated ion concentrations

In females from the Winnipeg River, urine free Ca^{2+} was highest in early-late vitellogenic fish and lowest in spawning fish (Table 5). Similarly, spawning females had very low bound Ca^{2+} concentrations. In females, the ratio of urine free:total Ca^{2+} was higher in early vitellogenic and spawning fish than in pre-vitellogenic and post-spawn fish. In males, urine free Ca^{2+} and free:total Ca^{2+} increased in maturing fish compared to immature fish, although bound Ca^{2+} was not statistically different. Urine was contaminated by seminal fluid in many of the spawning males, therefore it was not used in analyses. Similarly to urine free and bound Ca^{2+}, total Ca^{2+} was lowest in spawning females, and did not change between immature and maturing males. Urine PO_4^{3-} showed a similar trend to plasma PO_4^{3-}, with increasing concentrations in females up to mid-late vitellogenic fish, although values were not statistically different. Urine Ca^{2+}: PO_4^{3-} ratios were higher in early vitellogenic females than spawning females, with PO_4^{3-} at much higher concentrations than Ca^{2+} for both female and male fish at all reproductive stages.

Female urine F^- decreased in spawning fish similar to patterns seen in plasma F^-, and was at fairly similar concentrations to plasma F^- in most reproductive stages. Female urine Cl^- did not change between reproductive stages, and was always at much lower concentrations in the urine than plasma. Female urine Br^- increased in spawning fish relative to pre-vitellogenic fish. Female urine SO_4^{2-}, Na^+, K^+ and osmolality did not change between reproductive stages. Female urine Mg^{2+} was lower in spawning fish than in early and mid-late vitellogenic fish. Male urine F^-, Cl^-, Br^-, SO_4^{2-}, Na^+, Mg^{2+}, and osmolality did not change between those reproductive stages that were assessed (Table 5). Urine K^+ was about two times greater in maturing males than immature males.

The monovalent ions (Cl^-, Br^-, Na^+) were generally at greater concentrations in the plasma than in the urine, with the exception of

Table 5
Mean (±S.E.) urine Ca^{2+} and PO_4^{3-} concentrations (mM) from lake sturgeon captured in the Winnipeg River, Manitoba, Canada.

Sex	R. stage	*n*	Free Ca^{2+}	Bound Ca^{2+}	Free:Total Ca^{2+}	Total Ca^{2+}	PO_4^{3-}	Ca^{2+}:PO_4^{3-}
F	PV	8	0.68 ± 0.11^{ab}	0.25 ± 0.03^{c}	0.70 ± 0.05^{a}	0.93 ± 0.10^{b}	4.59 ± 0.61	0.15 ± 0.02^{ab}
F	EV	7	1.25 ± 0.21^{b}	0.07 ± 0.03^{ab}	0.95 ± 0.02^{b}	1.31 ± 0.22^{b}	6.66 ± 0.99	0.19 ± 0.03^{b}
F	M-LV	11	1.00 ± 0.20^{b}	0.16 ± 0.05^{bc}	0.84 ± 0.05^{ab}	1.22 ± 0.21^{b}	8.60 ± 2.52	0.14 ± 0.02^{ab}
F	SP	17	0.47 ± 0.03^{a}	0.03 ± 0.02^{a}	0.97 ± 0.02^{b}	0.50 ± 0.04^{a}	4.48 ± 0.32	0.11 ± 0.01^{a}
F	PS	6	0.90 ± 0.24^{ab}	0.26 ± 0.04^{c}	0.75 ± 0.05^{a}	1.16 ± 0.26^{b}	4.97 ± 1.15	0.18 ± 0.02^{ab}
M	IM	11	0.70 ± 0.14^{A}	0.20 ± 0.04	0.72 ± 0.06^{A}	0.90 ± 0.13	5.76 ± 0.94	0.13 ± 0.02
M	MA	8	1.06 ± 0.11^{B}	0.12 ± 0.05	0.91 ± 0.03^{B}	1.19 ± 0.12	7.63 ± 1.03	0.15 ± 0.02

R = reproductive, PV = pre-vitellogenic, EV = early vitellogenic, M-LV = mid-late vitellogenic, SP = spawning, PS = post-spawn, IM = immature, MA = maturing. Different superscript lowercase or uppercase letters indicate significant differences between female or male reproductive groups. One-way ANOVA for females, student's *t*-test for males, except for Manitoba female free Ca: non-parametric Kruskall–Wallis test followed by Dunn's multiple comparison tests ($P<0.05$).

F^- which was at similar concentrations, and K^+ which was at greater concentrations in the urine. The divalent (SO_4^{2-}, Mg^{2+}) and trivalent (PO_4^{3-}) ions were at greater concentrations in the urine than in the plasma, with the exception of Ca^{2+}. However, osmolality was always much higher in the plasma than in the urine.

3.5. Sex steroid concentrations

In female fish from the Winnipeg River, mid-late vitellogenic fish had greater plasma E_2 concentrations than any other reproductive stage (Fig. 5A). In females from the Lake Winnebago system, plasma E_2 peaked in the spawning fish, reaching concentrations that were 4-fold greater than at any other stage (Fig. 5B). For both populations, males had virtually no detectable E_2. In contrast, males had very similar patterns and magnitudes of increase in 11-KT and T in both populations, with both hormones increasing in maturing and spawning stages compared to immature fish (Fig. 5C–F). Females from both populations had much lower concentrations of androgens than males, and plasma concentrations of T and 11-KT increased with vitellogenesis and decreased post-spawning.

3.6. Calcified structures

Pectoral fin rays did not change in Ca^{2+} concentration between vitellogenic and non-vitellogenic reproductive stages in females (Fig. 6A). However, lateral scutes increased in Ca^{2+} percentage in vitellogenic fish as compared to non-vitellogenic fish, and showed a trend for decreasing mass (Fig. 6B).

4. Discussion

Life in a FW environment for cartilaginous fishes is a comparatively uncommon biological trait which is perhaps not so surprising given the role of Ca^{2+} in stabilizing ion flux (Wood, 1992), and its importance during the reproductive cycle (Mommsen and Korsgaard, 2008). Therefore, its regulation would seem of critical importance to this group of fishes, and particularly for a species such as lake sturgeon, which inhabits FW throughout its life cycle and has a reproductive strategy whereby a considerable investment of energy and demand for Ca^{2+} is required during reproduction (Peterson et al., 2007). FW elasmobranch fish are another example of FW cartilaginous vertebrates, however, this group maintains total plasma Ca^{2+} concentrations around 3–4 mM, and the primitive fishes, many with regressive cartilaginous endoskeletons, maintain plasma Ca^{2+} concentrations near 1.5–3 mM (Table 6). Therefore regulation of Ca^{2+} in the primitive fishes is low in comparison with teleosts which maintain total plasma Ca^{2+} from 2–5 mM (Holmes and Donaldson, 1969; Dacke, 1979).

As a group, sturgeons have very low circulating Ca^{2+} concentrations (Table 6), indeed, some of the lowest circulating Ca^{2+} concentrations in vertebrates, suggesting that they may be functioning close to a physiological threshold (Urist and Van de Putte, 1967). In addition, the largely cartilaginous skeleton of sturgeon, would suggest that those species which inhabit FW throughout their life would have a more limited capacity for Ca^{2+} storage. Therefore, the low circulating concentrations of Ca^{2+} and the relatively small reservoir of available Ca^{2+} suggests that lake sturgeon have evolved unique adaptive modifications for Ca^{2+} balance.

Ca^{2+} exists in three fractions in the plasma: free, as an ion-complex with ions such as PO_4^{3-}, and that which is bound to proteins (Dacke, 1979). Free Ca^{2+} is the most active form and is strictly regulated in most vertebrates (Dacke, 1979). A surprising result of this study on lake sturgeon was the finding that plasma free Ca^{2+} concentration increased over two-fold in mid-late vitellogenic female lake sturgeon from the Winnipeg River. A similar magnitude of increase has been reported previously for this species in response to osmotic stress (LeBreton and Beamish, 1998). Aberrations in the regulation of free Ca^{2+} have also been found in teleosts (Guerreiro et al., 2002). Indeed, the regulation of free Ca^{2+} in the plasma of many fish species is known to be less stringent than observed in other vertebrate groups (Dacke, 1979).

Another unique aspect of Ca^{2+} regulation in sturgeons may be a very high ratio of free to bound Ca^{2+}, as plasma concentrations approaching 99% free Ca^{2+} have been reported in juvenile Adriatic sturgeon, *A. naccarii* (Fuentes et al., 2007). However, in the present study, plasma free Ca^{2+} ranged from 56–72% in lake sturgeon (Fig. 3E,F). It is possible that the anadromous life history of *A. naccarii* does not necessitate the use of Ca^{2+} binding proteins to the degree that would be required to retain much lower concentrations of Ca^{2+} in the permanent FW environments inhabited by lake sturgeon. Primitive fishes have been reported to have lower concentrations of bound Ca^{2+} than more derived groups of fishes, such as teleosts (Urist et al., 1972). However, in teleosts, the ratio of free to total Ca^{2+} is close to 50% (Björnsson and Haux, 1985), which is similar to pre-vitellogenic females and immature male lake sturgeon in the present study.

4.1. Ca^{2+} regulation during reproduction

The highest concentrations of free, protein-bound, and total Ca^{2+} are found in teleosts during the female reproductive cycle. Total Ca^{2+} has been reported to range from 2.5–25 mM, with the excess Ca^{2+} bound to VTG (Urist and Van de Putte, 1967). In female lake sturgeon, free and bound Ca^{2+} increased during reproduction, and as a result, total Ca^{2+} increased up to 4 mM. Previous studies on sturgeons have identified a close correlation between total Ca^{2+} and VTG (Doroshov et al., 1997; Linares-Casenave et al., 2003).

4.2. Ca^{2+} uptake and mobilization

Elevated concentrations of Ca^{2+} during reproduction result from increased uptake and internal mobilization in fishes (Persson et al., 1994; Persson et al., 1998). The mechanisms for Ca^{2+} uptake are relatively unknown in FW cartilaginous fishes, but in sturgeons they are likely similar to what is known for teleosts. The teleost model of Ca^{2+} uptake in the gill involves passive entry of Ca^{2+} via apical channels (Hwang and Lee, 2007) due to low intracellular concentrations (Flik, 1997). Uptake appears to occur in mitochondria-rich cells and

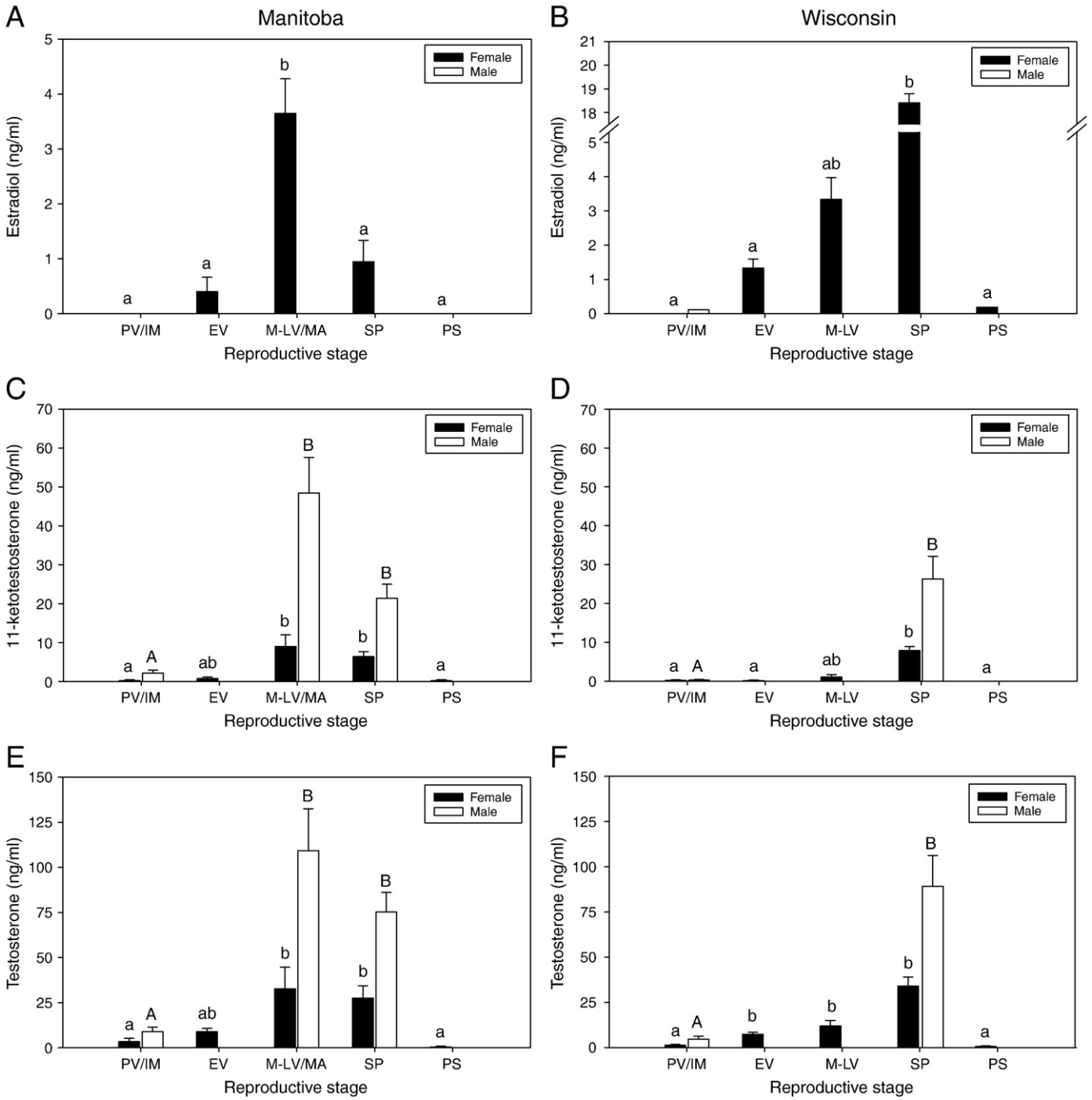


Fig. 5. Mean (+S.E.) plasma testosterone (A, B), 11-ketotestosterone (C, D) and estradiol (E, F) in lake sturgeon captured in the Winnipeg River, Manitoba, Canada and Lake Winnebago system, Wisconsin, USA. Different lowercase or uppercase letters indicate significant differences between female (black bars) or male (white bars) reproductive groups, respectively. One-way ANOVA, Tukey's multiple comparison tests, except for Manitoba males: non-parametric Kruskall–Wallis with Dunn's multiple comparison tests, $P<0.05$, $n=5$–23.

pavement cells (Shahsavarani and Perry, 2006), where Ca^{2+} is bound to proteins and transported to the basolateral membrane where it is actively pumped into the blood by Ca^{2+}-specific-ATPases or exchanged for Na^+ in Na^+-Ca^{2+} exchangers facilitated by the Na^+ gradient created by Na^+, K^+-ATPase (Hwang and Lee, 2007). A second source of Ca^{2+} uptake is in the gastrointestinal tract where Ca^{2+} uptake may occur by similar means, by vesicular-mediated transport, or by paracellular pathways (Khanal and Nemere, 2008). Intestinal Ca^{2+}-uptake is related to food uptake regardless of environment Ca^{2+} concentrations, and to drinking rate (Flik, 1997; Guerreiro et al., 2002).

Internal Ca^{2+} mobilization is achieved through bone resorption, which is controlled by the activity of osteoclasts (Dacke, 1979), which are known to be stimulated by hypercalcemic hormones such as parathyroid hormone-related-protein (PTHrP; Rottlant et al., 2005). In lake sturgeon, comparatively few internal Ca^{2+} sources are available if bony tissue is considered the only Ca^{2+} store. Fuentes et al. (2007) did find that overall Ca^{2+} concentrations of *A. naccarii* were comparable to slightly lower than those of teleost fishes, and presumably concentrated in the exoskeleton. Calcified plates in the head, fin, spines and rays, and five rows of scutes or ganoid scales are

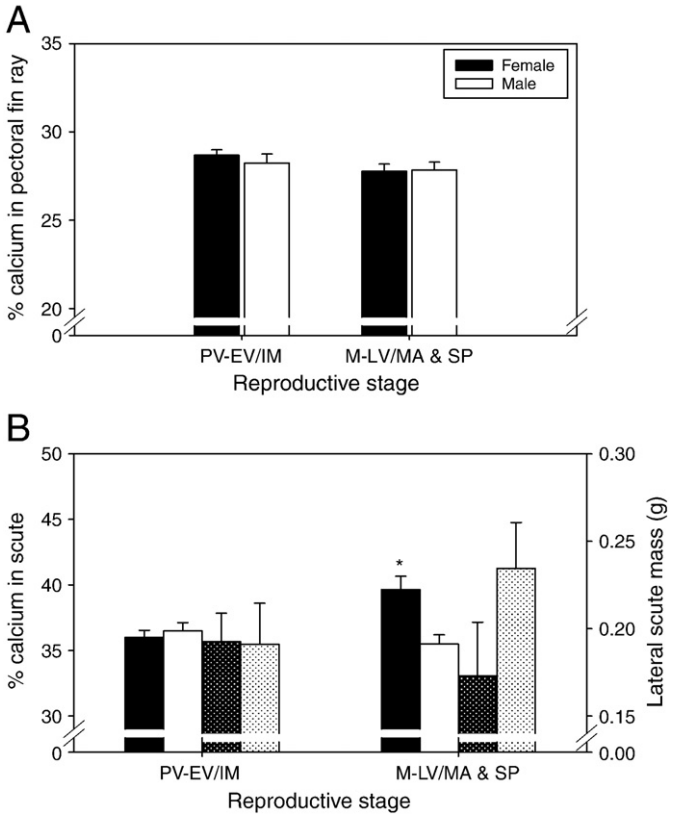


Fig. 6. Mean (±S.E.) pectoral fin ray percent Ca^{2+} (A), lateral scute percent Ca^{2+} and scute mass (B) from combined reproductive stages in lake sturgeon captured in the Lake Winnebago system, Wisconsin, USA. In (B) solid bars represent % Ca^{2+} and dotted bars represent scute mass. Females are designated with black bars and males with white bars. Percent Ca^{2+} is the mass of Ca^{2+} divided by total fin ray or scute mass. An asterisk indicates a significant difference ($P<0.05$) between reproductive groups within a sex, Student's *t*-test, $n=10–47$.

among the more prominent bony structures in sturgeons (Findeis, 1997). While resorption of the former two structures will likely pose significant challenges to skeletal integrity, resorption of the scutes is possible particularly given the resorption of Ca^{2+} from scales in bony fishes during vitellogenesis (Persson et al., 1994; Armour et al., 1997; Persson et al., 1998). In female lake sturgeon, this appears to be true as well, as calcified structures collected from the Lake Winnebago system sturgeon population indicated that mineral resorption may be occurring from some calcified structures, notably the scutes, but apparently not to a large degree in other structures such as the pectoral fin rays. Similarly, (Carragher and Sumpter, 1991) found that in rainbow trout, scales were resorbed but not internal bones after administration of E_2. In female lake sturgeon, interestingly, percent Ca^{2+} actually increased concomitant with a trend for decreasing scute mass. Thus, Ca^{2+} may be deposited in the scutes due to high circulating concentrations, or another mineral, such as PO_4^{3-}, is being preferentially resorbed.

4.3. Ca^{2+} excretion and renal handling

As plasma Ca^{2+} concentrations increase during reproductive development, Ca^{2+} excretion, via the urine, is also regulated. In the spawning female lake sturgeon, this is evident in the decrease of free, bound and total Ca^{2+} in the urine, likely due to a need to conserve remaining Ca^{2+} after the substantial output into oocytes has ceased. In lake sturgeon, bound Ca^{2+} is much lower in the urine than the

Table 6
Serum, plasma and urine Ca^{2+}, $Mg^{2+}$and PO_4^{3-} concentrations (mM) in freshwater-acclimated cartilaginous and primitive fishes.

Species	Fluid	Ca^{2+}	Mg^{2+}	PO_4^{3-}	Reference
Cephalospidomorphi					
Petromyzon marinus (AM)	S	2.4	1.6	1.30	Urist and Van de Putte, 1967
P. marinus (A)	S	2.2–2.7	1.8–2.0		Reviewed by Morris, 1980
P. marinus (A)	S	2.60, 1.87[UF], 1.74[F]		1.31, 1.17[UF]	Urist, 1963
Chondrichthyes					
Carcharhinus leucas (J)	P	3.0	1.3		Pillans et al., 2005
C. leucas (A)	S	3.0, 1.8[UF]	2.0	4.0	Urist, 1962
Dasyatis sabina	P	4.3			Piermarini and Evans, 1998
Potamotrygon spp.	S	3.0	2.3	1.29	Griffith et al., 1973
Potamotrygon spp.	S	3.6	1.78	6.7[a]	Thorson et al., 1967
Pristis microdon	B			3.1	Smith and Smith, 1931
	U	1.7	1.3	4.5	
P. perotteti	S	4.15	0.85		Thorson, 1967
Sarcopterygii					
Lepidosiren paradoxa	S	2.0	1.3	2.2	Urist et al., 1972
Neoceratodus forsteri	S	1.6	1.2	1.4	Urist et al., 1972
Protopterus annectens	S	1.8	1.1	7.0[b]	Urist et al., 1972
Actinopterygii–Chondrostei–Acipenseriformes					
Acipenser baerii	S	2.4	3.2		Natochin et al., 1985
	U	0.8	1.2		
A. baerii	P	1.2			Rodriguez et al., 2002
A. fulvescens	P	1.28	1.25	4.8[a]	Magnin, 1962
A. fulvescens	P	1.2[F]			LeBreton and Beamish, 1998
A. fulvescens	P	1.8–2.1, 1.1–1.4[F]	0.6–1.0	1.7–3.7	This study[c]
	U	0.9, 0.7[F]	1.5	4.6–5.8	
A. guldenstadti	S	2.4	1.0		Natochin et al., 1985
	U	2.45	0.75		
A. medirostris	P	2.15[d]	0.72		Potts and Rudy, 1972
A. naccarii	P	1.25–1.5			Fuentes et al., 2007
A. nudiventris	S		1.55		Natochin et al., 1985
	U				
A. oxyrinchus	P	1.85	0.85	5.7[a]	Magnin, 1962
A. ruthenus	S	2.5	0.9		Natochin et al., 1985
	U	0.85	0.7		
A. stellatus	S	2.8	1.05		Natochin et al., 1985
	U				
A. sturio	P	2.25	1.27	3.07	Magnin, 1962
	U	1.26	0.77	6.63	
A. transmontanus	S	1.8	2.0	3.3	Urist and Van de Putte, 1967
A. transmontanus	P	1.59(A), 1.96(J)			McEnroe and Cech, 1987
	U	0.27(A)			
Huso huso	S	2.2	1.05		Natochin et al., 1985
	U	1.2	0.6		
Polyodon spathula	S	2.2	1.7	3.3	Urist et al., 1972
Actinopterygii–Chondrostei–Polypteriformes					
Erpetoichthys calabaricus	S	2.00	1.64	1.80	Urist et al., 1972
Polypterus weeksii	S	2.31	1.0	1.24	Urist et al., 1972
Actinopterygii–Holocephali					
Amia calva	S	2.9	1.2	1.5	Urist et al., 1972
Lepisosteus platostomus	S	2.6	1.8	2.2	Urist et al., 1972

A Adult, AM = ammoceote, J = juvenile, B = Blood—not stated in reference whether plasma or whole blood, P = plasma, S = serum, U = urine, UF = ultrafilterable fraction, F = free.
[a] Phosphorous in meq.
[b] Hemolyzed blood.
[c] Pre-vitellogenic females and immature males from the Winnipeg River, Manitoba and the Lake Winnebago system, Wisconsin.
[d] Fish acclimated to freshwater for 3 days.

plasma presumably due to glomerular filtration, with free Ca^{2+} ranging from 70 to 97% (Table 3). The role of the kidneys in Ca^{2+} regulation has received relatively little study in fishes, and considering the importance of renal reabsorption of Ca^{2+} in higher vertebrates, this may be a fruitful area for future studies. Interestingly, Butler and Alia Cadinouche (1995) found that renal reabsorption was important to the regulation of Mg^{2+} and PO_4^{3-} but not Ca^{2+} in stanniectomized North American eels *Anguilla rostrata*. A recent study found that in primitive fishes, renal Ca^{2+} reabsorption is quite high (Patel et al., 2009). In general, very little is known in regards to renal Ca^{2+} handling in primitive FW fishes (Wright, 2007), especially in relation to changes in environmental Ca^{2+}, and in sturgeons.

4.4. PO_4^{3-} and associated ion regulation

Ca^{2+} and PO_4^{3-} concentrations are closely correlated in vertebrates due to their roles in the ossification of bone, and specifically in the formation of mineralized calcium phosphate or hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Compared to Ca^{2+} regulation, PO_4^{3-} regulation and its interaction with Ca^{2+} in fishes has received less attention, with few studies conducted in relation to seasonal reproductive changes (Srivastava and Srivastava, 1994). In fishes, PO_4^{3-} is an important component of VTG (Mommsen and Walsh, 1988). Vitellogenic females therefore, have high concentrations of plasma Ca^{2+} and PO_4^{3-} which must be fueled by increased external Ca^{2+} uptake primarily via the gills (Flik et al., 1985), to some degree via the intestine depending on drinking rate (Guerreiro et al., 2002), and by the resorption of internal calcified and phosphate-rich structures (Carragher and Sumpter, 1991; Persson et al., 1994; Armour et al., 1997; Persson et al., 1998; Yamada et al. 2002). In female lake sturgeon, plasma PO_4^{3-} increases with reproductive stage until spawning, where it drops precipitously, consistent with its role in vitellogenesis. Urine PO_4^{3-} also shows the same general trend. In teleosts, PO_4^{3-} is regulated by intestinal uptake primarily from dietary sources, and is excreted by renal routes (Renfro, 1997). In FW teleosts, urine excretion rates of PO_4^{3-} reflect extracellular concentrations, increasing when PO_4^{3-} is in internal excess and decreasing when it is limited (Vielma and Lall, 1998). The ratio of plasma Ca^{2+}: PO_4^{3-} reveals that Ca^{2+} is still elevated in spawning fish. This time lag or decoupling between Ca^{2+} and PO_4^{3-} may be due to a more gradual decline in VTG production and a continued up-regulation of Ca^{2+} transport into the body from ambient water sources, while bone/calcified tissue resorption, a source of PO_4^{3-} (Yamada et al., 2002), has already ceased.

Several other ions were also related to reproductive stage. Mg^{2+} and F^- showed the most similar patterns of increase to Ca^{2+}. Both ions increased in the plasma during vitellogenic reproductive stages (and spawning for Mg^{2+}), and decreased in spawning fish in the urine, similar to Ca^{2+}. Mg^{2+}, like Ca^{2+}, is a divalent cation, and binds to VTG (Björnsson and Haux, 1985; Mommsen and Walsh, 1988). Unlike Ca^{2+}, the primary source of Mg^{2+} uptake is via the gastrointestinal tract, with the gill uptake playing a secondary role, although it is primarily excreted by the kidneys, similar to Ca^{2+} (Bijvelds et al., 1998). It is also stored in calcified tissues, although its endocrine regulation is less well understood (Bijvelds et al., 1998). F^- was the only anion showing a distinct pattern with reproductive stage, suggesting that F^- may be associated with some of the elevated concentrations of cations such as Ca^{2+} and Mg^{2+}. Indeed, F^- is known to form ion complexes in solution, such as: $Ca_5(PO_4)_3F$, CaF_2 and MgF_2, and may be stored in bone tissue at high concentrations in fishes (Camargo, 2003).

The principal monovalent ions Na^+ and Cl^- did not appear to play a large role in vitellogenesis, as they changed very little with reproductive stage and were in much greater concentration in the plasma than the urine. Other monovalent ions such as Br^-, F^-, and K^+ were at lower, similar or higher concentrations in the urine, respectively. The divalent and trivalent ions were regulated quite differently, with higher concentrations in the urine than plasma, with the exception of Ca^{2+}, similar to other sturgeon species (Table 6). Regulation of ions in the urine of FW teleosts is generally similar, although Mg^{2+} may be higher in the plasma than the urine (Hickman and Trump, 1969).

4.5. Ca^{2+} regulation in male lake sturgeon

In males, changes in free and total Ca^{2+} were minor in magnitude, compared to the changes in females with reproductive stage. Changes in total Ca^{2+} were not due to bound Ca^{2+}, which did not change, and it is not surprising considering that concentrations of VTG would not be expected to increase in wild males. The slight increases in free Ca^{2+} in maturing and spawning males in the Winnipeg River and total Ca^{2+} in spawning fish of both populations may be due to Ca^{2+} needs of developing testes. In support of this, scale resorption and gonad Ca^{2+} concentrations have been found to be elevated in maturing Atlantic salmon (Persson et al., 1998). Similar to females, plasma PO_4^{3-} decreases in spawning fish are likely due to physiological mechanisms that limit the loss of PO_4^{3-} once germinal development is complete. In the urine, the significant increase in the ratio of free:total Ca^{2+} in maturing males is due to increases in free Ca^{2+}.

4.6. Endocrine aspects of Ca^{2+} regulation

The sex steroids play a role in the regulation of Ca^{2+} uptake and resorption, as well as in reproductive development and maturation. In females, gonadotropin I is released from the pituitary and stimulates the production of T in ovarian thecal cells. T is then transported to ovarian granulosa cells where it is aromatized to E_2, and subsequently released into the blood (Nagahama, 1983; Nagahama and Yamashita, 2008). E_2 acts directly on the hepatocytes, causing the synthesis and release of VTG (Mommsen and Walsh, 1988).

E_2 has also been shown to have a hypercalcemic role in Ca^{2+} homeostasis. Estrogen receptors have been located in fish scales and bone, where E_2 has been shown to mobilize Ca^{2+} and PO_4^{3-} from scales (Armour et al., 1997). E_2 has also been shown to increase environmental uptake of Ca^{2+} through the gills and intestine (Guerreiro et al., 2002). In the lake sturgeon, the increases in E_2, 11-KT and T concentrations at different reproductive stages are consistent with that found in the other species of sturgeons (Webb et al., 2002; Webb and Erickson, 2007; Wildhaber et al., 2007), and previously in lake sturgeon, although fish were not identified according to their reproductive stage (McKinley et al., 1998). The increases of E_2 also coincided with changes in scute Ca^{2+} content, although the actual increase in Ca^{2+} content which coincided with a trend for a decrease in mass may be due to an E_2-induced overcompensation in Ca^{2+} mobilization resulting in increased Ca^{2+} turnover, and subsequent incorporation into internal stores (Persson et al., 1994).

Hormonal control of Ca^{2+} regulation is known to involve a number of hormones in fishes, notably staniocalcin, calcitonin, PTHrP, vitamin D, and prolactin. Corpuscles of Stannius, which secrete staniocalcin, a hypocalcemic hormone, are not known to be present in sturgeons (Sasayama, 1999). The hypocalcemic hormone, calcitonin, has not been measured in sturgeons, although its influence on Ca^{2+} regulation is ambiguous in fishes (Takei and Loretz, 2006). However, there is evidence that it may be related to the female reproductive cycle (Watts et al., 1975) or post-feeding Ca^{2+} suppression (Sasayama, 1999). PTHrP, a hypercalcemic peptide hormone, has been shown to increase plasma Ca^{2+} in *A. naccarii* via increased influx and decreased efflux (Fuentes et al., 2007). PTHrP, like E_2, is known to stimulate Ca^{2+} resorption in internal calcified structures such as scales (Rottlant et al., 2005). In sturgeons, vitamin D concentrations have been found to be low, suggesting that the capacity to metabolize and store vitamin D may be limited (Urist and Van de Putte, 1967). Furthermore, the efficacy of vitamin D may be limited, as administration of

vitamin D_3 to *A. transmontanus* acclimated to either FW or seawater had no effect on plasma Ca^{2+} concentrations (McEnroe and Cech, 1994). However, administration of ovine prolactin did elevate plasma Ca^{2+} concentrations in seawater acclimated *A. transmontanus* (McEnroe and Cech, 1994).

4.7. Environmental differences in regulation of Ca^{2+}

Water chemistry was very different between the two populations of lake sturgeon sampled. The higher Ca^{2+}, PO_4^{3-} and pH in the Lake Winnebago system compared to the Winnipeg River, corresponded with differences in internal Ca^{2+} and PO_4^{3-} regulation. Plasma concentrations of free Ca^{2+} in pre-vitellogenic females and immature males were both very similar between the populations, although bound Ca^{2+} was greater in the Winnipeg River fish (Fig. 3A–D). Therefore, fish in the Winnipeg River appear to be compensating for lower ambient Ca^{2+} concentrations by maintaining a greater protein-bound-fraction, even prior to vitellogenic stages. Furthermore, during vitellogenesis, females from both populations respond to increased Ca^{2+} demands with elevated plasma free and bound Ca^{2+}, although with a much greater response in fish in the Winnipeg River. When compared at the level of total Ca^{2+}, differences in Winnipeg River fish were great enough to distinguish different female reproductive stages, whereas in the Lake Winnebago system, only vitellogenic from non-vitellogenic females could be distinguished (Fig. 3G,H). Therefore, under low environmental Ca^{2+} concentrations, there appears to be an up-regulation of the mechanisms for mobilization or uptake of Ca^{2+}. In support, elevated Ca^{2+} uptake has been found in FW fishes acclimated to low Ca^{2+} concentrations (Flik et al., 1986; Mol et al., 1999). In teleost fishes, increased Ca^{2+} needs appear to be met through an up-regulation of gill epithelial Ca^{2+} channels (Shahsavarani and Perry, 2006; Liao et al., 2007). Other factors such as diet, obviously affect Ca^{2+} handling, although the dietary differences between the populations are not known. Fish in the Lake Winnebago system are larger at the same reproductive stages, and may have faster growth rates due to diet quality and/or quantity, water temperature differences, and latitudinal differences in growing season.

Similar to the relationship for plasma Ca^{2+}, PO_4^{3-} was elevated in the Winnipeg River fish, remaining two-fold greater regardless of reproductive stage. Increased uptake under low ambient concentrations has also been found for phosphorous in teleosts (Mol et al., 1999), which is directly related to internal PO_4^{3-} concentrations (Avila et al., 2000). The differences in PO_4^{3-} resulted in quite different ratios of Ca^{2+}: PO_4^{3-} between populations, with the Winnipeg River fish maintaining more plasma PO_4^{3-} than free Ca^{2+}, and the Lake Winnebago system fish maintaining similar free Ca^{2+} to PO_4^{3-} (Fig. 4C,D). Renal clearance may be a large factor in the differences in Ca^{2+} and PO_4^{3-} concentrations between the two populations, as sturgeon have been documented with higher urine Ca^{2+} concentrations in hypercalcemic environments (Natochin et al., 1985; McEnroe and Cech, 1987). Environmental pH differences may also affect Ca^{2+} regulation between the two populations, but little is known as to the effects of moderately-elevated pH on Ca^{2+} flux rates in fishes. Overall, these relationships suggest that the fish preferentially retain Ca^{2+} and PO_4^{3-} at low environmental concentrations.

Concentrations of sex steroids were remarkably similar between populations at similar reproductive stages. The only exception was that of E_2 in spawning females, which reached a much higher concentration in the Lake Winnebago system fish. The difference may be due to the difference in timing of sampling between the systems. However, this increase is interesting, particularly when considered with lower plasma bound Ca^{2+} concentrations found in the same population. This would seem to indicate that the magnitude of increase in E_2 is not directly responsible for the amount of VTG in circulation. E_2 influences a multitude of factors during vitellogenesis and oocyte maturation, and therefore the functional significance of the greater plasma peak in the Lake Winnebago system fish is unclear.

5. Conclusions

Although FW cartilaginous fishes are relatively uncommon, this life history strategy poses interesting challenges for Ca^{2+} homeostasis. In the lake sturgeon, Ca^{2+} handling has some unique characteristics in comparison to what is currently known for teleosts and for many other vertebrates. Free Ca^{2+} concentrations were not strictly maintained during the reproductive cycle, with a two-fold increase in plasma concentrations at the peak of vitellogenesis. Sturgeons lack corpuscles of Stannius, which exert anti-hypercalcemic control in teleosts, and may be a reason for this marked increase in Ca^{2+}. Both free and total Ca^{2+} are regulated at very low concentrations in the lake sturgeon, and in sturgeons in general, representing some of the lowest concentrations known in vertebrates. Bound Ca^{2+} increases during the reproductive cycle as expected with binding to VTG, but the amount of bound Ca^{2+} in non-reproductive fish is not as low as has been previously reported in sturgeons. However, in low Ca^{2+} environments bound Ca^{2+} concentrations were maintained at greater concentrations than in higher Ca^{2+} environments in the lake sturgeon, suggesting that in very high Ca^{2+} environments, bound Ca^{2+} concentrations may be maintained at low concentrations. Plasma PO_4^{3-} showed a similar inverse relationship relative to environmental concentrations. Unfortunately relatively little is known in regards to the mechanisms for Ca^{2+} uptake, mobilization, and excretion in lake sturgeon and other FW cartilaginous fishes, and their endocrine control. Future research into these areas is merited, especially considering the unique aspects of Ca^{2+} regulation in lake sturgeon.

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